MALACOLOGIA, 2012, 55(2): 263–284

LIGHT AND TRANSMISSION ELECTRON MICROSCOPY OF TWO SPERMATOGENIC PATHWAYS AND UNIMORPHIC SPERMATOZOA IN *VENUSTACONCHA ELLIPSIFORMIS* (CONRAD, 1836) (BIVALVIA: UNIONOIDA)

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ABSTRACT

This study followed the typical and atypical pathways of spermatogenesis in *Venustaconcha ellipsiformis* (Conrad, 1836) (Bivalvia: Unionoida: Unionidae), during an entire year and documents the developmental stages of both using light and transmission electron microscopy. The spermatozoa produced by both pathways were essentially morphologically identical and contained a single acrosomal vesicle. Production of spermatozoa in the two pathways overlapped from May to October, and vitellogenic eggs were present over this same time period. Atypical spermatogenesis also occurred from November through April. The typical pathway involved the mitotic and meiotic divisions that include spermatogonial cells, primary and secondary spermatocytes, spermatids and mature spermatozoa. In contrast, atypical spermatozoa appeared to be produced in a pathway that included spermatozoa morulae and spermatids produced from spermatogonial cells located in Sertoli cells. Large amorphous inclusions (AI) observed in Sertoli cells, and apparently acting as secondary lysosomes in digesting morulae, are described for the first time. The morphology of the AI was variable, with the AI sometimes containing structures that were organelle-like in appearance and some of the AI appeared to merge together. Phagocytes associated with both the typical and atypical pathways, but distinct from Sertoli cells, were observed clearing the acini of cellular debris. Immunoelectron microscopy detected the presence of both female-transmitted (F) and male-transmitted (M) mitochondria in the spermatozoa present in July (when fertilization takes place). We propose that the necessity of producing spermatozoa with F and M mt genome-bearing mitochondria, to maintain dioecy and 50 : 50 sex ratios in freshwater bivalve populations, accounts for the > 200 million year maintenance of two spermatogenic pathways in the Unionoida.

Key words: atypical and typical spermatogenesis, doubly uniparental inheritance of mitochondrial DNA (DUI), ultrastructure, Unionoida, *Venustaconcha ellipsiformis*.

INTRODUCTION

The Mollusca is one of several higher taxa in which heteromorphic spermatozoa are found (Buckland-Nicks & Chia, 1986; Jespersen et al., 2001; Buckland-Nicks & Thompkins, 2005). Outside the Mollusca, other taxa that are known to produce heteromorphic male gametes are plants, arthropods, annelids, nematodes, echinoderms and vertebrates (Cook & Wedell, 1999; Ferraguti et al., 2002; Till-Bottraud et al., 2005; Paccagnini et al., 2006; Immler, 2008). Many gastropod species produce spermatozoa by two spermatogenic pathways referred to as typical and atypical (Dohmen, 1983). In the typical pathway, fertilizing spermatozoa develop by a series of mitotic and meiotic cell divisions involving spermatogonial cells, primary and secondary spermatocytes, spermatids and mature spermatozoa. Other spermatogenic pathways that result in non-fertilizing spermatozoa or in which spermatozoa are produced by stages that differ from the typical pathway stages, are called atypical. Spermatozoa produced by an atypical pathway are often morphologically distinct from typical spermatozoa (Heard, 1975). When present together, the two morphologically distinct spermatozoa are usually referred to as eusperm or fertilizing spermatozoa (typical) and parasperm, or non-fertilizing spermatozoa (atypical) (Healy & Jamieson, 1981). Functions for the atypical spermatozoa

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have been reported as aiding in protection and transport of the typical spermatozoa to the egg, often by forming a spermatozeugmata (Nishiwaki & Tochimoto, 1969; Lynn, 1994; Cook & Wedell, 1999; Ishibashi et al., 2000; Jespersen et al., 2001; Ferraguti, et al., 2002), providing nutrition to the egg, creating a hostile environment for competing spermatozoa (Gould, 1917; Kura & Nakashima, 2000; Buckland-Nicks & Thompkins, 2005; Immler, 2008) or interfering with female ability to be reinseminated with rival spermatozoa (Cook & Wedell, 1999; reviewed in Till-Bottraud et al., 2005). Spermatozeugmata have also been reported as containing atypical spermatozoa (Nishiwaki & Tochimoto, 1969; Waller & Lasee, 1997), although some spermatozeugmata contain fertilizing spermatozoa (Ó Foighil, 1989). Various pathways of spermatogenesis have been described for species that produce heteromorphic spermatozoa including separate spermatogonial cells (Buckland-Nicks & Thompkins, 2005), one type of spermatogonial cell (Ferraguti et al., 2002; Paccagnini et al., 2006) and accessory cells (Reinke, 1902), while in some cases, the pathway is not stated (Cook & Wedell, 1999; Jespersen et al., 2001).

As is noted in Buckland-Nicks & Chia (1986), several terms have been used to describe the somatic cells that are located on the wall of a testicular acinus and which extend into its lumen. In the present study, in agreement with Syasina (1992), whose review is on molluscs, these cells will be referred to as "Sertoli cells". Sertoli cells have been associated with spermatogenesis in many different animals (Roosen-Runge, 1977; Reunov et al., 2004; Smita et al., 2005; Sasso-Cerri et al., 2006; Wang et al., 2006; reviewed in Syasina, 1992), including molluscs. Their roles in molluscs have been noted as that of nursery cells, which provide nutrients to developing spermatozoa, phagocytosis and destruction of residual bodies and abnormal or unspawned spermatozoa and contributions to hormonal control of spermatogenesis and maintenance of the blood-testis barrier (Buckland-Nicks & Chia, 1986; Pipe, 1987; Sousa et al., 1989; Dorange & LePennec, 1989; Rocha & Azevedo, 1990; Eckelbarger & Davis, 1996; Erkan & Sousa, 2002; Syasina, 1992; Kiyomoto et al., 1996). Sertoli cells have also been documented as containing spermatozoa morulae (Matos et al., 1998). Spermatozoa morulae, which are reported to initially contain spermatids (Matos et al., 1998), or possibly various cells of the typical spermatogenic process (Chatchavalvanich et al., 2006), are widespread in the Unionoida (Coe & Turner, 1938; Bloomer, 1946; Heard, 1975; Jones et al., 1986; Kotrla, 1988, 1989; Haggerty et al., 1995; Byrne, 1998; Garner et al., 1999; Shiver, 2002; Cek & Sereflisan, 2006; Chatchavalvanich et al., 2006). Spermatozoa morulae in unionoid bivalves have been observed in species representing all unionoid families and they are always associated with the atypical pathway of spermatogenesis (Heard, 1975, unpublished observations; Kotrla, 1988, 1989).

Bivalve species in the order Unionoida typically possess separate sexes (dioecy) and two highly unusual reproductive characteristics: high fidelity doubly uniparental inheritance of mitochondrial (mt) DNA (DUI; Breton et al., 2007) and two distinct spermatogenic pathways (Heard, 1975; Kotrla, 1988, 1989). DUI is a unique form of mtDNA transmission that has been detected only in mytiloid, veneroid and unionoid bivalves (Breton et al., 2007). Under DUI, females pass on their F-type mitochondrial DNA (mtDNA) to both sons and daughters. This is similar to transmission occurring under strict maternal inheritance of mtDNA (SMI) in all other animal species. However, in contrast to the situation present in other animals, male mussels transmit their M-type mtDNA, via the five mitochondria comprising the spermatozoon mid-piece, only to sons. Male somatic tissue typically contains predominantly the mother's F-type mtDNA, whereas male gametes exclusively contain the M-type mtDNA inherited from the father. The strict preservation of this mode of inheritance in dioecious unionoid species has produced two parallel but highly divergent mtDNA lineages that originated > 200 million years ago (Breton et al., 2007, 2011; Doucet-Beaupré et al., 2010). The DUI system in bivalves not only challenges our traditional view of strict maternal inheritance of mtDNA, it also demonstrates remarkable genetic novelties in animal mt genomes: an extension (M*COX2e*) of the cytochrome *c* oxidase subunit II protein (M*COX2*) that is only encoded by the unionoid bivalve M mt genomes and two additional, gender-specific and rapidly evolving mt-encoded proteins (i.e., protein genes other than the typical set of 13 usually found in animal mtDNAs), whose functions remain unknown (the F*ORF* and M*ORF* proteins in the unionoid F and M mt genomes, respectively). An antibody to the extension region of the M*COX2* protein and an antibody to the F*ORF* protein have been produced (Chakrabarti, 2006, 2007; Breton et al., 2009, 2011).

In addition to the presence of DUI in dioecious unionoid species, morulae, which are associated with the atypical pathway of spermatogenesis, are present in the testicular tissues of all species that have been thoroughly studied, as cited above. The genesis and function of these structures, broadly distributed within the Unionoida, are unclear but previous authors have stated that these structures develop into mature spermatozoa (Kotrla, 1988; Matos et al., 1998). Therefore, the phenomena of DUI and two distinct spermatogenic pathways, typical and atypical, are inextricably linked to unionoid spermatogenesis.

Phagocytic and lysosomal activities associated with spermatogenic tissues are common. As mentioned above, Sertoli cells have been observed engaged in these processes in various animal taxa (Reunov et al., 2004; Smita et al., 2005; Sasso-Cerri et al., 2006; Wang et al., 2006), including molluscs (Buckland-Nicks & Chia, 1986; Pipe, 1987; Dorange & LePennec, 1989; Sousa et al., 1989; Rocha & Alzevedo, 1990; Syasina, 1992; Eckelbarger & Davis, 1996; Kiyomoto et al., 1996; Erkan & Sousa, 2002). Phagocytes that are distinct from Sertoli cells are well documented in a variety of organisms including leeches (Bonet & Molinas, 1991), caecilians (Smita et al., 2005), lugworms (Pacey & Bentley, 1992), sea urchins (Reunov et al., 2004), and molluscs (López et al., 1997).

Spermatozoon ultrastructure has been considered a useful character for evolutionary comparisons with acrosomal ultrastructure being of particular interest. For example, studies between spermatozoa of *Velesunio ambiguous* (Philippi, 1847) (Unionoida: Hyriidae) and trigonioid spermatozoa (*Neotrigonia bednalli* [Verco, 1907]) established similarities of multiple vesicles within the acrosome of these two animals leading to the proposal that the Trigonioida and Unionoida could have a relatively close phylogenetic relationship (Healy, 1989). This phylogenetic hypothesis has been corroborated by subsequent studies (Hoeh et al., 1998, 2009; Graf & Cummings, 2006). Some freshwater clams produce spermatozoa with a single acrosomal vesicle and this characteristic is generally designated a plesiomorphic character state (Franzen, 1956; Trimble & Gaudin, 1975). However, acrosome development has been demonstrated to take many forms in bivalves (Reunov et al., 1999) and the acrosome structure itself could be related to aspects of egg-spermatozoa interaction during fertilization as well evolutionary history (Popham, 1979).

The present report describes, in detail, the typical and atypical pathways of spermatogenesis in *Venustaconcha ellipsiformis* (Conrad, 1836) and their temporal overlap with each other and with the presence of vitellogenic eggs. It is based on consecutive, monthly samples from an entire year using light and transmission electron microscopic observations. Additionally, immunoelectron microscopy (IEM) techniques demonstrated the presence of both the M and F mt proteins in unionoid spermatozoa. The results from the IEM experiments, together with the morphological evidence from the typical and atypical spermatogenic pathways, suggest a hypothesis for the > 200 million year persistence of dual spermatogenic pathways in unionoid bivalves.

MATERIALS AND METHODS

Specimens

Because it was known from preliminary studies that *Venustaconcha ellipsiformis* (Conrad, 1836) exhibited both typical and atypical spermatogenesis pathways and that frequent sampling was required by the aims of this study, this species was selected due to its proximity to Saginaw Valley State University. Monthly collections of *V. ellipsiformis* were made from the Sugar River in Gladwin County, Michigan, between January and December 2006, with the exception of March (Fig. 1). Animals were placed in a container of ice and immediately transported to the laboratory where male and female gonadal tissue was excised and processed as described below. All protocols complied with appropriate animal care committee policies at Saginaw Valley State University and adhered to the legal requirements established in the United States regarding animal use and care in research.

Conventional Transmission Electron Microscopy

Tissues were placed in 2.5% glutaraldehyde in mussel buffer (Misamore & Lynn, 2000). They were stored at 4°C for up to several months. Post fixation was in 0.5% OsO_4 in buffer for 1 hr, also at 4°C. Both fixatives were followed by 2 x 10 min buffer washes. Dehydration was in a graded series of acetone and tissues were embedded in Spurr's resin. Sections were cut at 70 nm, stained with uranyl acetate and lead citrate and viewed with a JEOL 1400 transmission electron microscope at 80 KV.

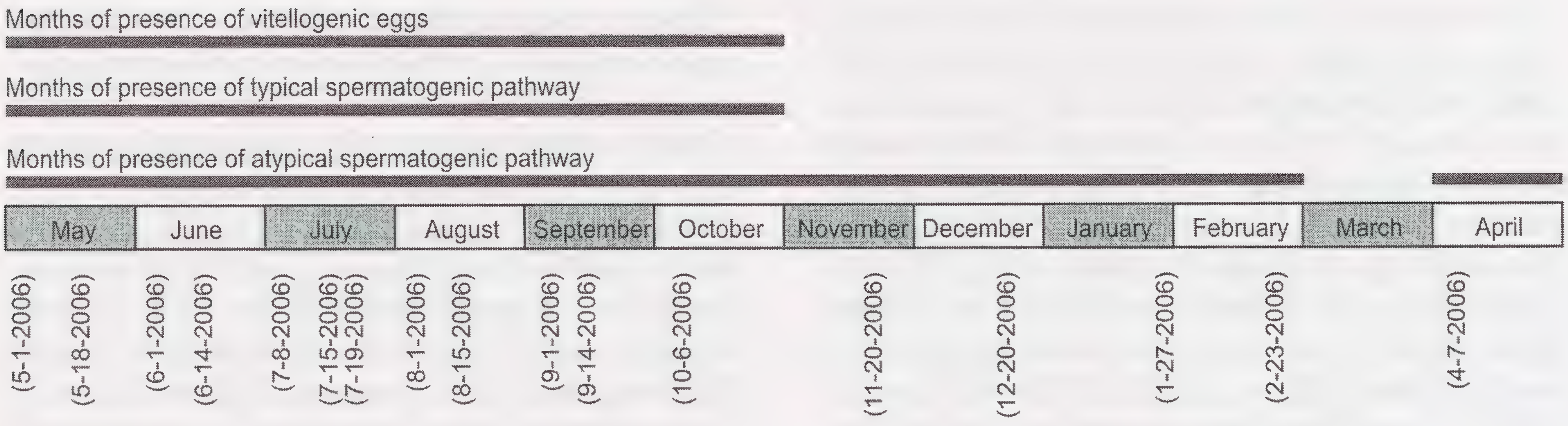


FIG. 1. Phenology of the presence of vitellogenic eggs and typical and atypical pathway sperm; note that no sample was taken in March. Sampling dates are in parentheses.

Light Microscopy

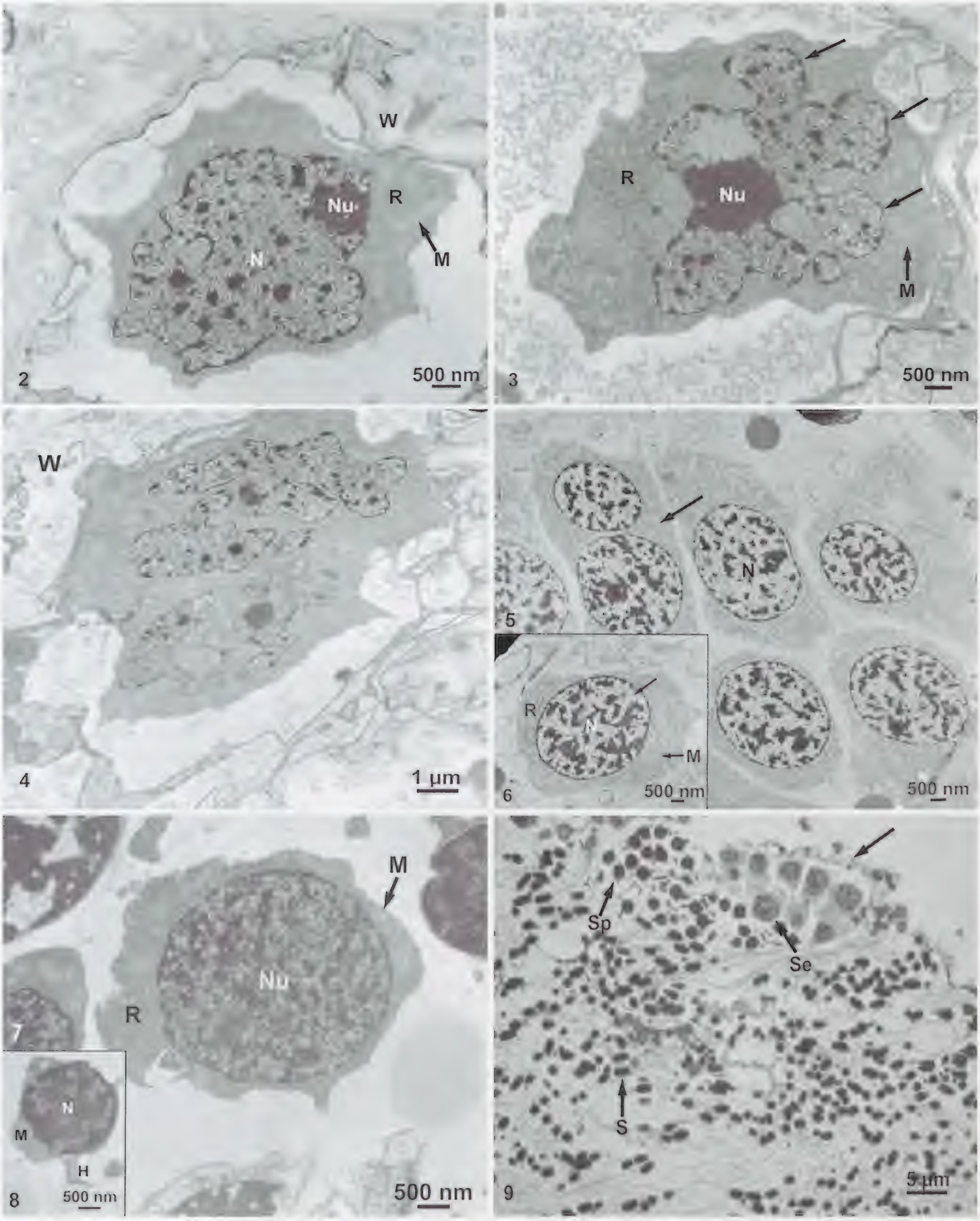
For some of the light microscopy, sections of the Spurr's embedded material were cut at 0.5 µm and stained with toluidine blue. Others were fixed with 1.5% buffered formalin and prepared for embedding in paraffin according to standard procedure (Humason, 1967). Sections were cut at a thickness of 7 µm and stained with the Feulgen's DNA-specific protocol and counter-stained with 0.5% fast green (Humason, 1967). Ovarian sections were stained with hematoxylin and eosin (Humason, 1967). Images were viewed under a Nikon Optiphot and collected using Image Pro 5.1.2 software.

Immunoelectron Microscopy

Bivalve tissues were fixed in 1.0% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 4 hr and washed 3 x 10 min in buffer and embedded in LR White. Tissues were sectioned (70 nm), placed on nickel grids and incubated for 15 min in a low molecular weight blocking solution (0.05% glycine in PBS), followed by 30 min in a high molecular weight blocking solution (goat block solution, Electron Microscopy Sciences, Fort Washington, Pennsylvania) and a 5 min wash in incubation buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7.4 with 0.2% BSA-c and 15 mM NaN_3). Sections were then incubated with either the M*COX2e* primary antibody (1:200) or the F*ORF* primary antibody (1 : 10) in incubation buffer for 1 hr. After 3 x 5 min incubation buffer washes, grids were placed in gold solution (10 nm gold in goat anti rabbit serum, diluted 1 : 20 in incubation buffer, Electron Microscopy Sciences) for 2 hr. Six x 5 min incubation buffer washes were followed by 3 x 5 min PBS washes. Sections were post fixed in 2.0% glutaraldehyde in PBS for 5 min, washed in PBS for 5 min x 5 in dH_2O for 2 min each. Sections were post stained in uranyl acetate for 15 min and lead citrate for 1 min and viewed with a JEOL 1400 or 100 CX TEM at 80 KV. Control IEM protocols omitted the primary antibodies.

Figure Abbreviations

A	acrosome
AI	amorphous inclusion
C	collar of midpiece
E	egg
ER	endoplasmic reticulum
F	flagellum
H	cytoplasmic hook
L	lipid
Lu	lumen
Ly	lysosome
M	mitochondrion
Mo	spermatozoon morula
N	nucleus
Nu	nucleolus
P	primary spermatocyte
Pc	phagocyte
R	ribosomes
S	spermatozoon
SC	Sertoli cell
SCl	spermatozoa cluster
Se	secondary spermatocyte
SG	spermatogonial cell
SGG	spermatogonial cell group
Sp	spermatid
St	stock
V	vacuole
W	acinus wall



FIGS. 2–9. Typical spermatogenic pathway. FIG. 2: Typical pathway spermatogonial cell at acinus wall; FIG. 3: Typical pathway multi-lobed (arrows) spermatogonial nucleus; FIG. 4: Typical pathway syncytium prior to break up into individual cells; FIG. 5: Typical pathway syncytium after break up into individual cells; division of cells (arrow) indicates the formation of primary spermatocytes and additional spermatogonial cells; FIG. 6: Typical pathway primary spermatocyte (arrow) with large, oblong nucleus; note less dense euchromatin (arrow) than in spermatogonial cell; FIG. 7: Typical pathway secondary spermatocyte with spherical nucleus containing highly condensed chromatin; FIG. 8: Typical pathway spermatid with mitochondria at one side of the nucleus; FIG. 9: Movement of typical pathway cells from acini wall (arrow); spermatogonial cells and primary spermatocytes are not in view.

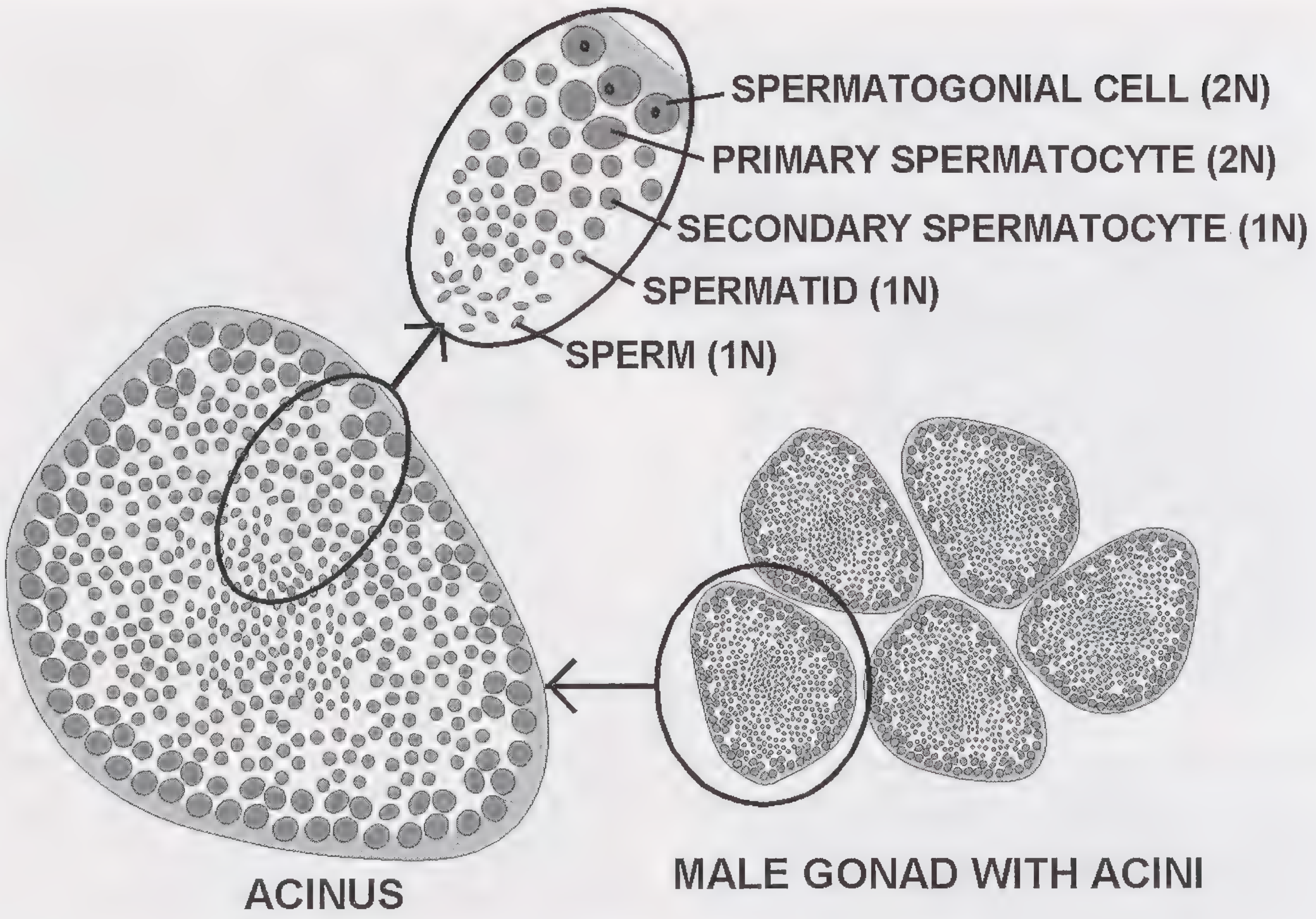


FIG. 10. Line drawing of typical pathway spermatogenesis compiled from several light and electron micrographs.

RESULTS

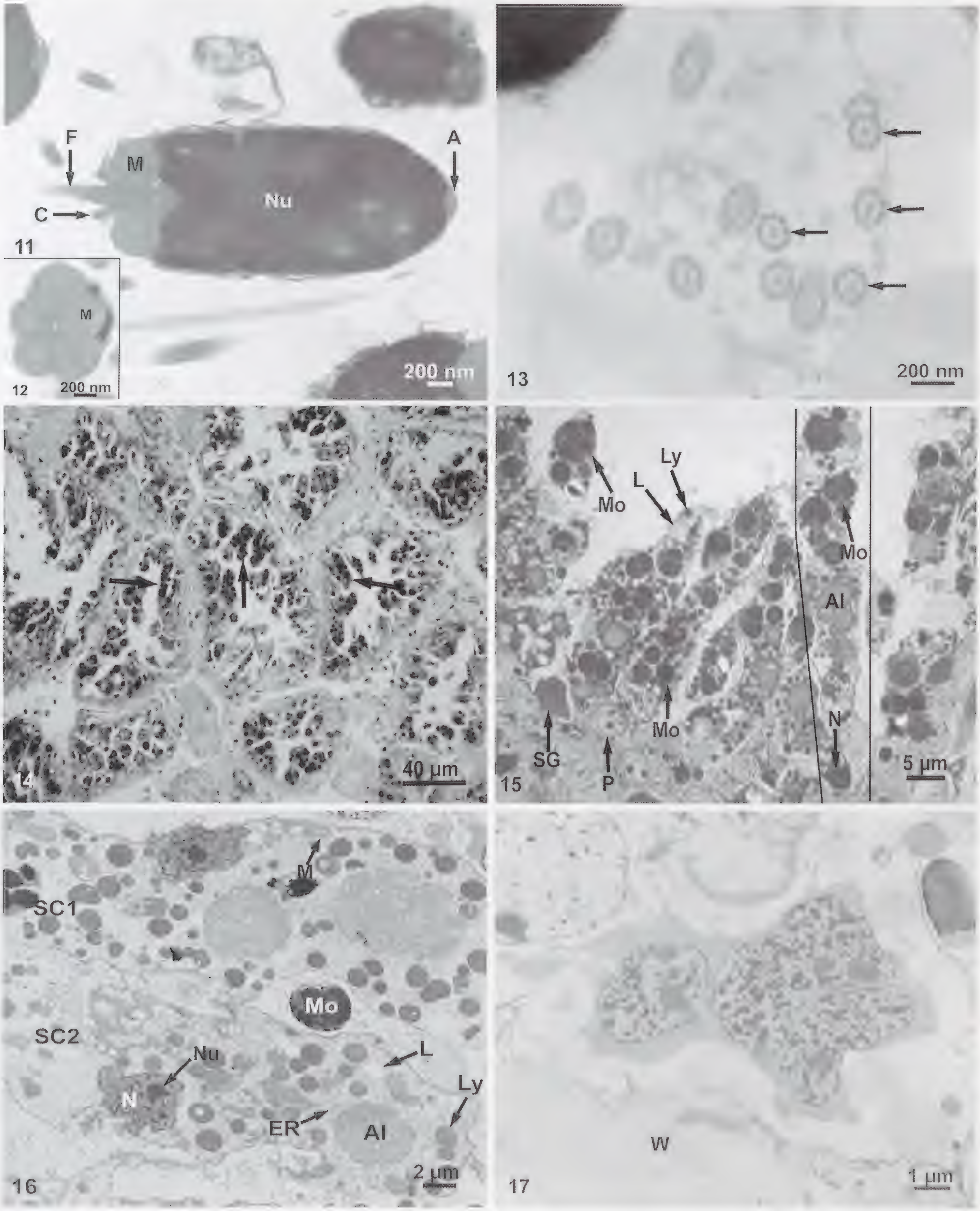
Typical Spermatogenic Pathway

The architecture of the testes in unionoid bivalves has been described elsewhere (Heard, 1975). In *Venustaconcha ellipsiformis*, typical spermatogenesis took place in the acini. The process began in early May and ended in October (Fig. 1). Spermatogenesis originated in spermatogonial cells, which were initially located on the acinar wall (Fig. 2). Spermatogonial cells contained a large, lobed nucleus with a prominent nucleolus, semi-condensed chromatin and dense euchromatin. Also present were numerous mitochondria and ribosomes (Fig. 2). Preceding division into more spermatogonial cells and primary spermatocytes, the spermatogonial nucleus became multilobed (Fig. 3). Newly formed spermatogonial cells and primary spermatocytes formed a syncytium, which eventually broke apart (Figs. 4, 5). Mature primary spermatocytes contained an oblong spherical nucleus, often with no obvious nucleolus, but containing evenly distributed condensed chromatin of varying amounts. Euchromatin in primary spermatocytes was distinctly less electron dense than that of the spermatogonial cells while mitochondria and ribosomes were numerous (Fig. 6). Secondary spermatocytes had less cytoplasm than primary spermatocytes and the nuclei were spherical with highly condensed chromatin content. Mitochondria and ribosomes remained (Fig. 7). In the spermatid stage, mitochondria were observed aggregated at one side of the nuclei and cytoplasmic hooks were common (Fig. 8). As typical spermatogenesis proceeded in May, spermatogonial cells, and all other cells produced from them, moved away from the acinar wall toward the acinar center (Fig. 9). In July, acini were filled with spermatozoa (Fig. 9). The entire process of typical spermatogenesis is summarized in Figure 10.

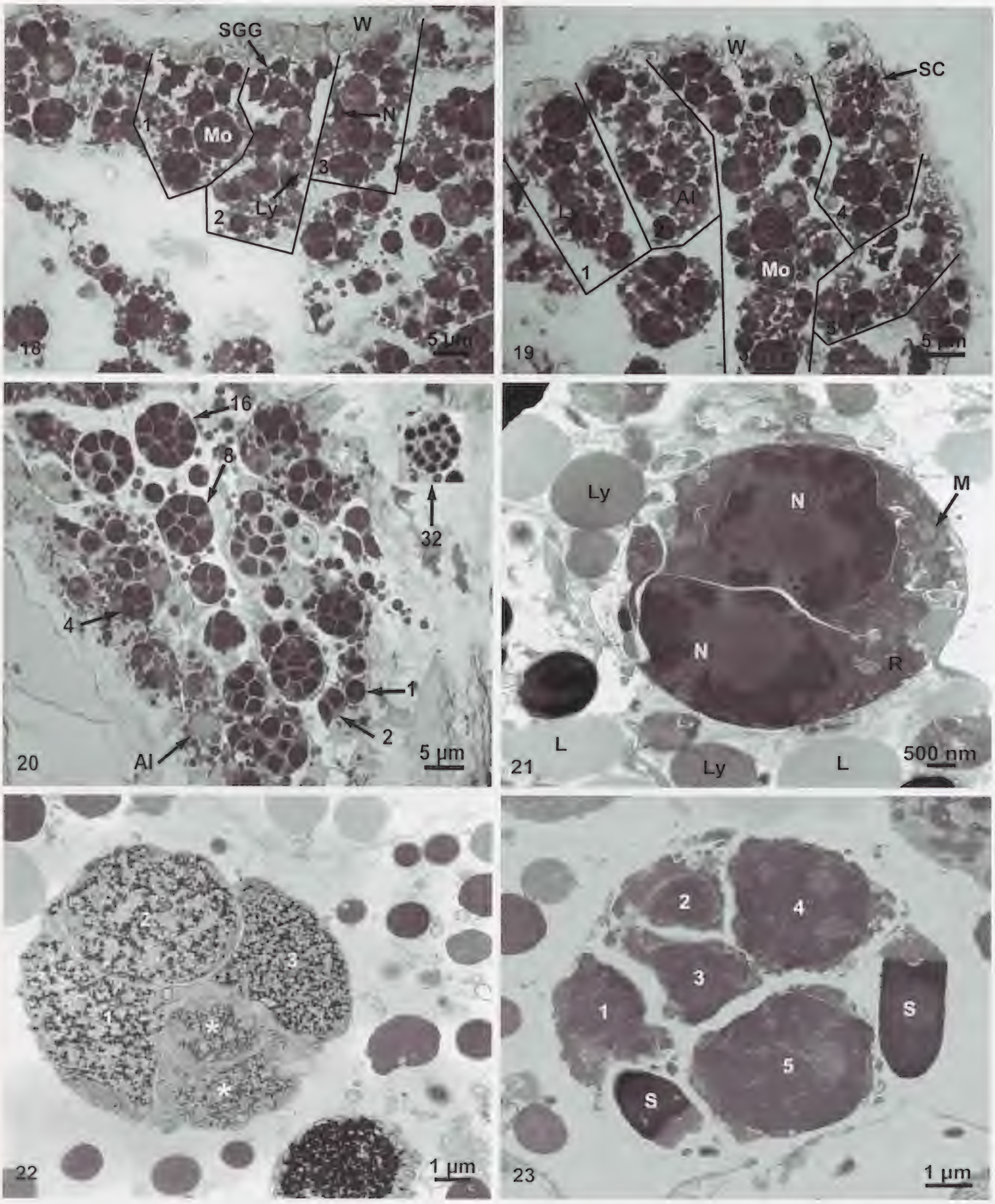
Mature spermatozoa had a bullet shaped nucleus of condensed chromatin with an acrosome containing a single vesicle (Fig. 11). Five mitochondria were observed at the posterior end of all spermatozoa studied (Fig. 12) and a single flagellum emerged through the collar of the mid-piece (Fig. 11). Axonemes contained the 9+2 arrangement of microtubules (Fig. 13). No spermatozeugmata (spermatozoa balls) were observed.

Atypical Spermatogenic Pathway and Sertoli Cells

The atypical pathway of spermatogenesis in *V. ellipsiformis* also took place in the acini but was active year round. The designation of spermatozoa observed from October – April as atypical was inferred from the lack of any



FIGS. 11–17. Typical and atypical spermatogenic pathways. FIG. 11: Typical pathway spermatozoon with bullet shaped nucleus; FIG. 12: Typical pathway spermatozoon showing five mitochondria in cross section of midpiece; FIG. 13: Cross sections of typical pathway spermatozoa axonemes showing 9+2 arrangement of microtubules (arrows); FIG. 14: Atypical pathway Sertoli cells (arrows) projected finger-like into the acini; FIG. 15: Atypical pathway Sertoli cells with one indicated inside lines; FIG. 16: Two atypical pathway Sertoli cells; FIG. 17: Atypical pathway syncytium prior to breakup into individual cells.



FIGS. 18–23. Atypical spermatogenic pathway. FIG. 18: Sertoli cells (1, 2) showing spermatogonial cell groups; FIG. 19: Later stage Sertoli cells (1, 2, 3, 4) showing high numbers of morulae and spermatozoa in clusters but absence of spermatogonial cells; FIG. 20: Atypical pathway morulae showing 1, 2, 4, 8, 16, and 32 (inset) cells; FIG. 21: Atypical pathway two-celled morula with highly condensed chromatin in the nuclei; FIG. 22: Atypical pathway four-celled morula with uncondensed chromatin in the nuclei inferring cell division within the morula; note that one of the four cells (*) had apparently begun its next division asynchronously to cells 1, 2 and 3; FIG. 23: Atypical pathway morula with five cells (1, 2, 3, 4, 5) and two mature spermatozoa (S) visible.

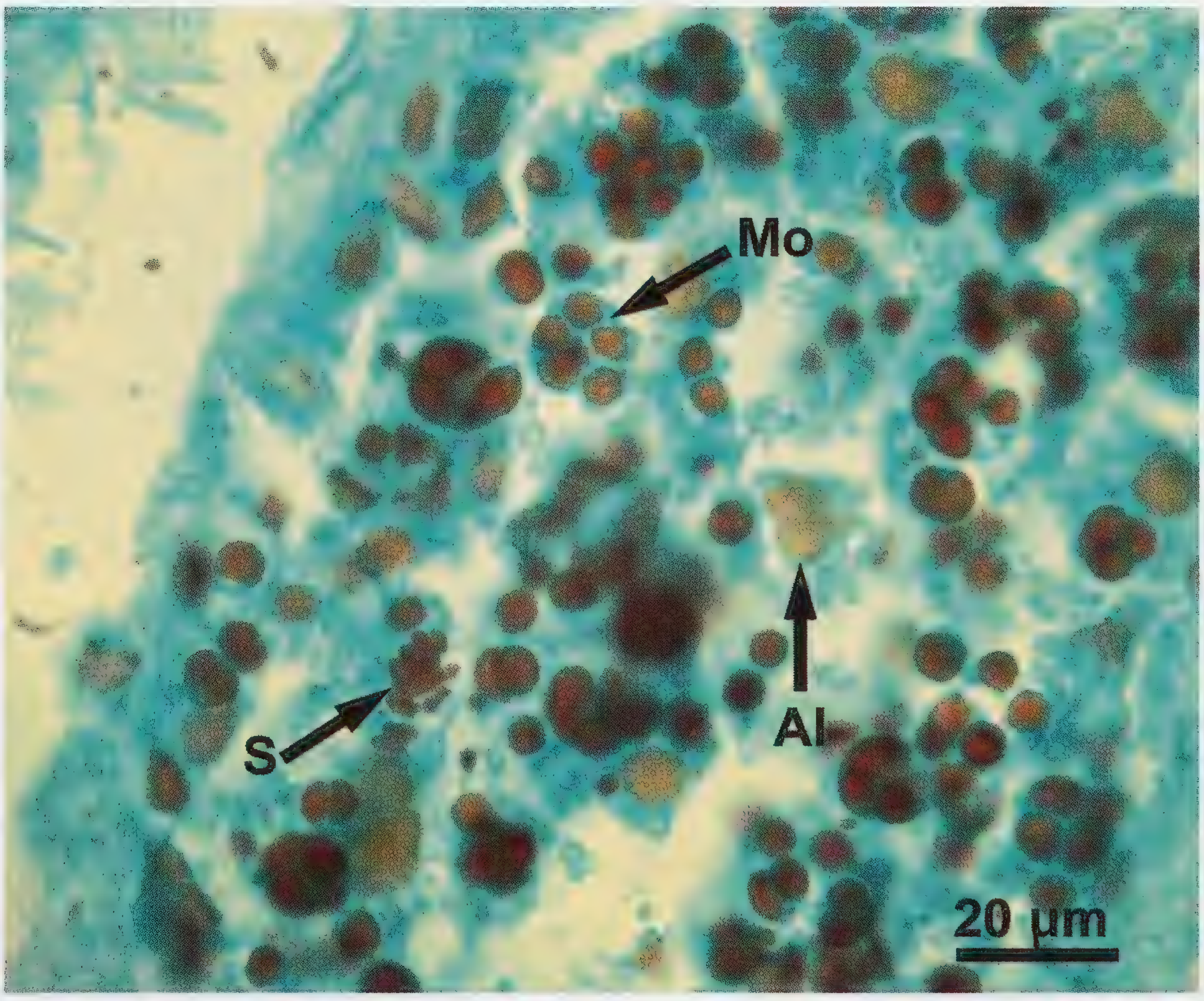


FIG. 24. Feulgen's stain on atypical pathway Sertoli cells; note positive (red) stain on morula, amorphous inclusion, and spermatozoa.

observations of activity in typical pathway spermatogonial cells beyond the occasional primary spermatocyte stage. A significant cell type involved in the atypical pathway was the Sertoli cell (Figs. 14–16). These cells were initially located at the acinar wall but developed into finger-like projections protruding into the acinar lumen as they matured (Figs.14, 15). Syncytia, essentially identical to those of the typical pathway, were observed in Sertoli cells near the acinar wall (Fig. 17). Sertoli cells contained nuclei with an undulating outline, a nucleolus and patches of condensed chromatin dispersed throughout the nuclei (Fig. 16). Sertoli cells also displayed mitochondria, endoplasmic reticulum, lipid bodies, lysosomes, sperm morulae (see below) and amorphous inclusions (AI) (see below) (Figs. 15–17). All of these structures were identified by their morphological characteristics. No long, thin cytoplasmic extensions were present (Figs. 15, 16), nor were junctions between cells. The syncytia broke apart into groups of separate spermatogonial cells (Fig. 18) essentially identical to those of the typical pathway (Fig. 2). Some Sertoli cells contained many morulae as well as spermatozoa clusters but no spermatogonial cells (Fig. 19, cells 1, 2, 3, 4).

Spermatozoa Morulae

Spermatozoa morulae were numerous in Sertoli cells (Figs. 15, 18, 19). Each morula contained 1, 2, 4, 8, 16 or 32 cells (Fig. 20). The numbers 8, 16 and 32 were assumed due to the thinness of the sections and the likelihood of cell division in the morulae (see below). Nuclei of the morulae cells had highly condensed chromatin with patches of lighter areas and mitochondria and ribosomes were present (Fig. 21). An intact membrane surrounded the individual cells of each morula as well as the entire morula (Figs. 21, 22). Many morulae were in close contact with lipid bodies and lysosomes (Fig. 21). Apparent stages of division of the cells in the morulae, inferred from the appearance of the uncondensed chromatin and smaller size of some of the cells, were observed (Fig. 22). All cells within a morula did not necessarily divide synchronously (Figs. 22, 23). The same Sertoli cell often contained morulae possessing different cell numbers (Figs. 15, 18, 19, 20).

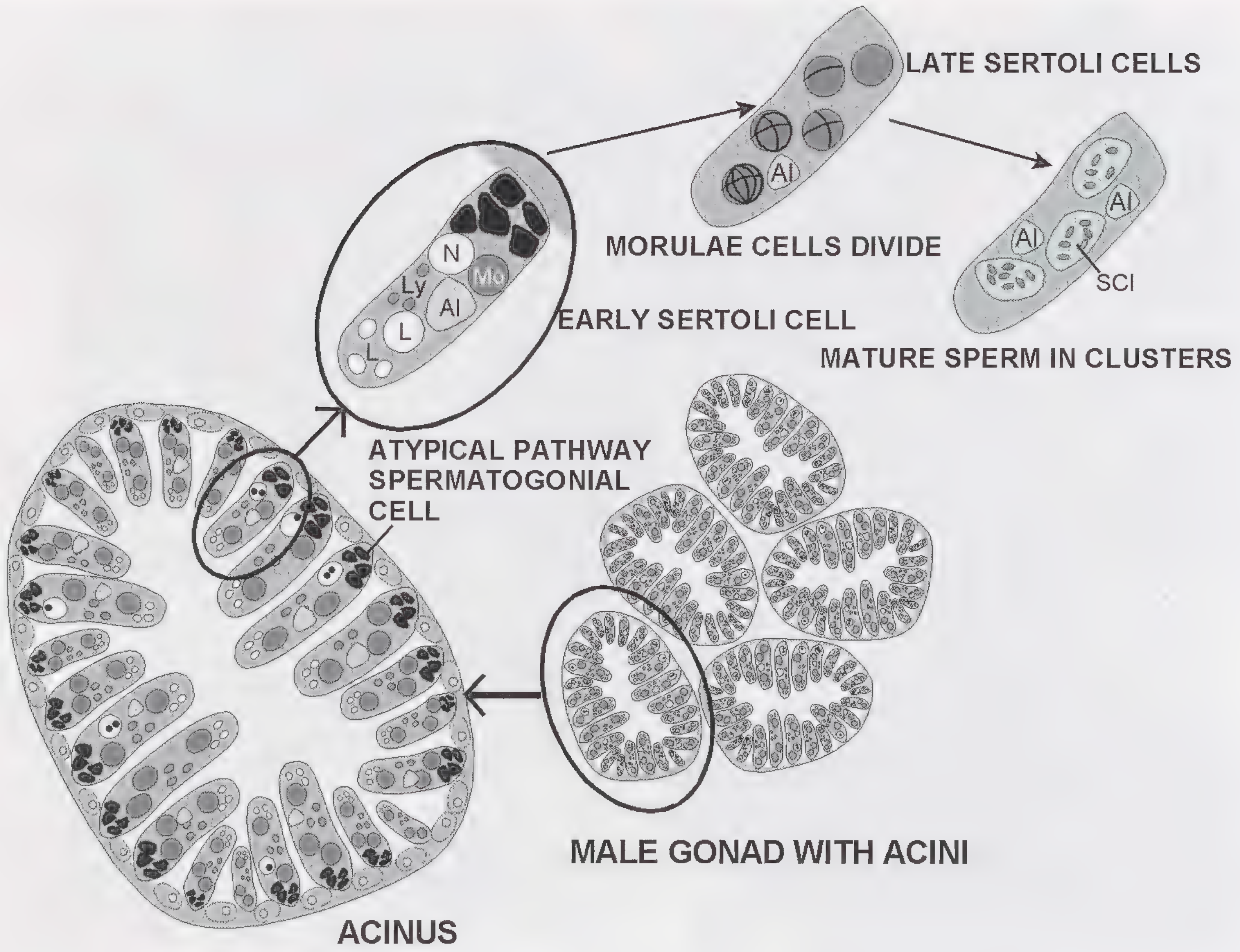


FIG. 25. Line drawing of atypical pathway spermatogenesis compiled from several light and electron micrographs.

Atypical Pathway Spermatozoa

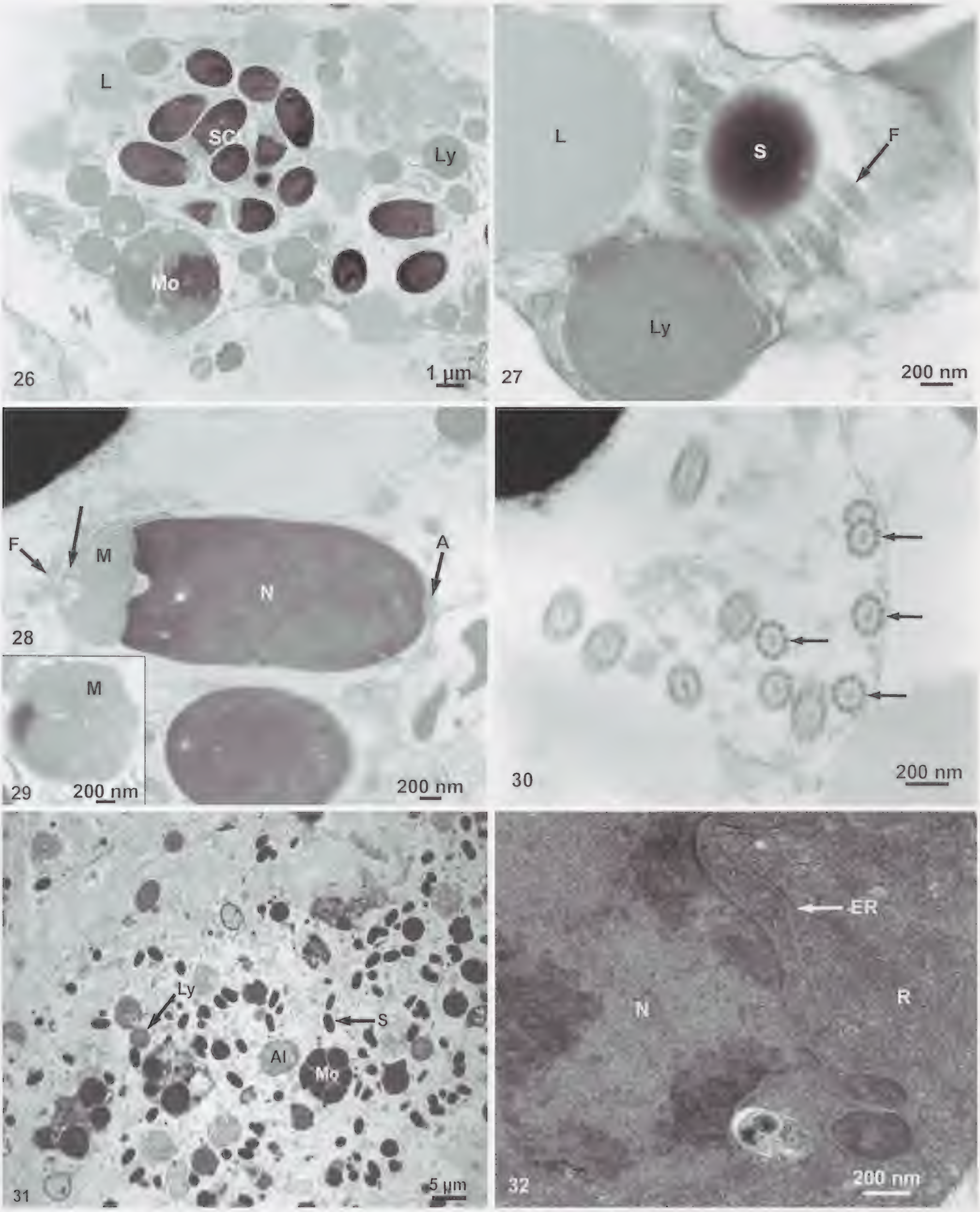
Mature atypical spermatozoa were often associated with the cells of a morula (Fig. 23) and were also located in membrane bound clusters in late stage Sertoli cells (Figs. 19, 24). The entire process of atypical spermatogenesis is summarized in Figure 25. The precise number of spermatozoa in clusters was difficult to ascertain because of the section thinness (Fig. 26). Atypical spermatozoa stained positive for DNA, as did morulae cell nuclei (Fig. 24). Flagella were observed wrapped around the spermatozoa when the spermatozoa were clustered (Fig. 27). Atypical spermatozoon structure closely matched that of the typical spermatozoa with a bullet shaped nucleus containing condensed chromatin, an acrosome with a single vesicle, five mitochondria in the mid-piece and a single flagellum which emerged through the collar of the mid-piece (Figs. 28, 29). Axonemes displayed the 9+2 configuration (Fig. 30). No spermatozeugmata (spermatozoa balls) were observed. Sertoli cells eventually broke down and released their contents into acinar lumina. At this stage, all of the various Sertoli cell organelles, morulae, AI (see below) and mature spermatozoa could be seen scattered in the acinar lumina (Fig. 31).

Amorphous Inclusions (AI)

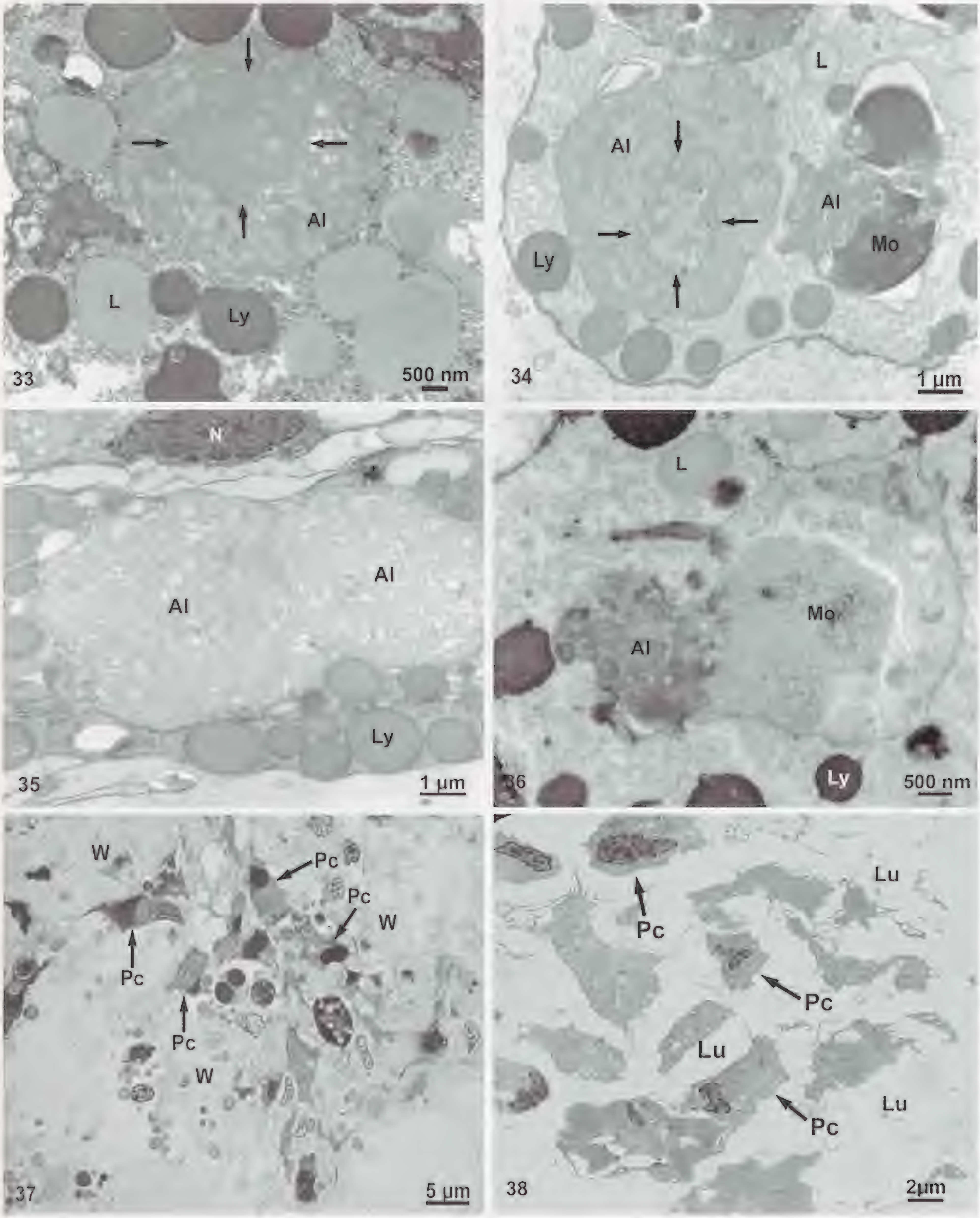
Sertoli cells typically contained structures designated as amorphous inclusions (AI). These bodies were of variable diameter and number in Sertoli cells (Figs. 15, 20, 24). At the ultrastructural level, an apparent metamorphosis of the internal structure of the inclusions was observed. Some AI displayed more distinct organelles such as mitochondria, rough endoplasmic reticulum and nuclei (Fig. 32) while others contained material reminiscent of a nucleus (Figs. 33, 34) or were primarily composed of amorphous material (Fig. 35). Lipids and lysosomes were observed in close contact with the AI (Figs. 33–35) and some AI appeared in close contact with each other (Fig. 35). Apparent transitional stages between morulae and AI were observed (Figs. 34, 36). The AI stained a yellowish brown color with Feulgen's stain (Fig. 24) and they had a continuous outer membrane (Figs. 33, 34).

Phagocytes

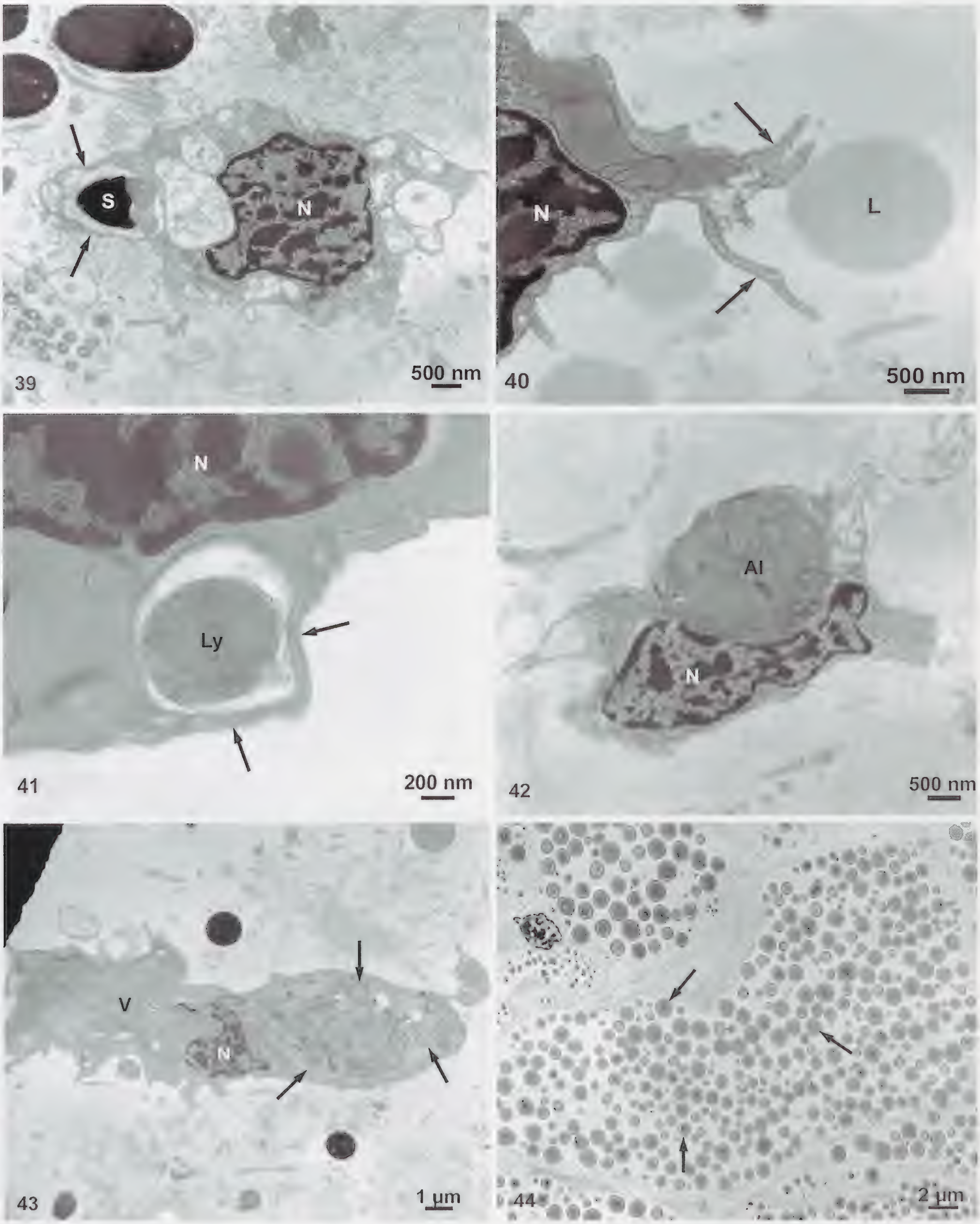
Cells recognized as phagocytes, based on similarities to the morphology and engulfing activity of cells from molluscs and other inver-



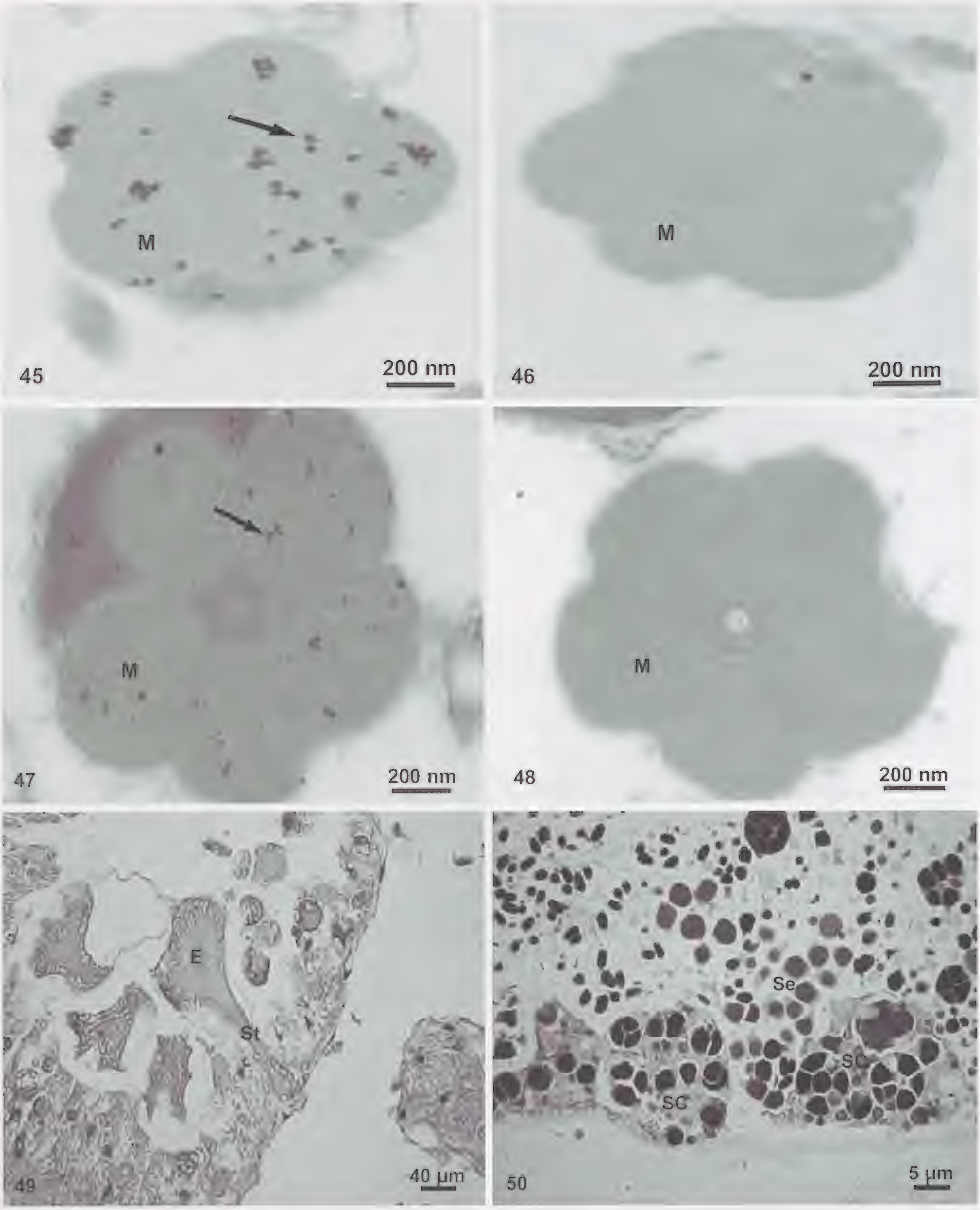
FIGS. 26–32. Atypical spermatogenic pathway. FIG. 26: Partial atypical pathway Sertoli cell containing spermatozoa clusters; FIG. 27: Atypical pathway spermatozoon showing how its flagellum wrapped around the spermatozoan; note that the spermatozoan is in cross section at the nuclear level; FIG. 28: Atypical pathway spermatozoon with bullet shaped nucleus; note flagellum fragment wrapped around spermatozoon; FIG. 29: Atypical pathway spermatozoon showing five mitochondria in cross section of midpiece; FIG. 30: Atypical spermatozoa axonemes in cross section; note two central microtubules (arrows); FIG. 31: Cell organelles and spermatozoa scattered in an acinus lumen as the Sertoli cells broke down in the atypical pathway; FIG. 32: Apparent early stage amorphous inclusion in Sertoli cell of atypical pathway; note the presence cellular organelles.



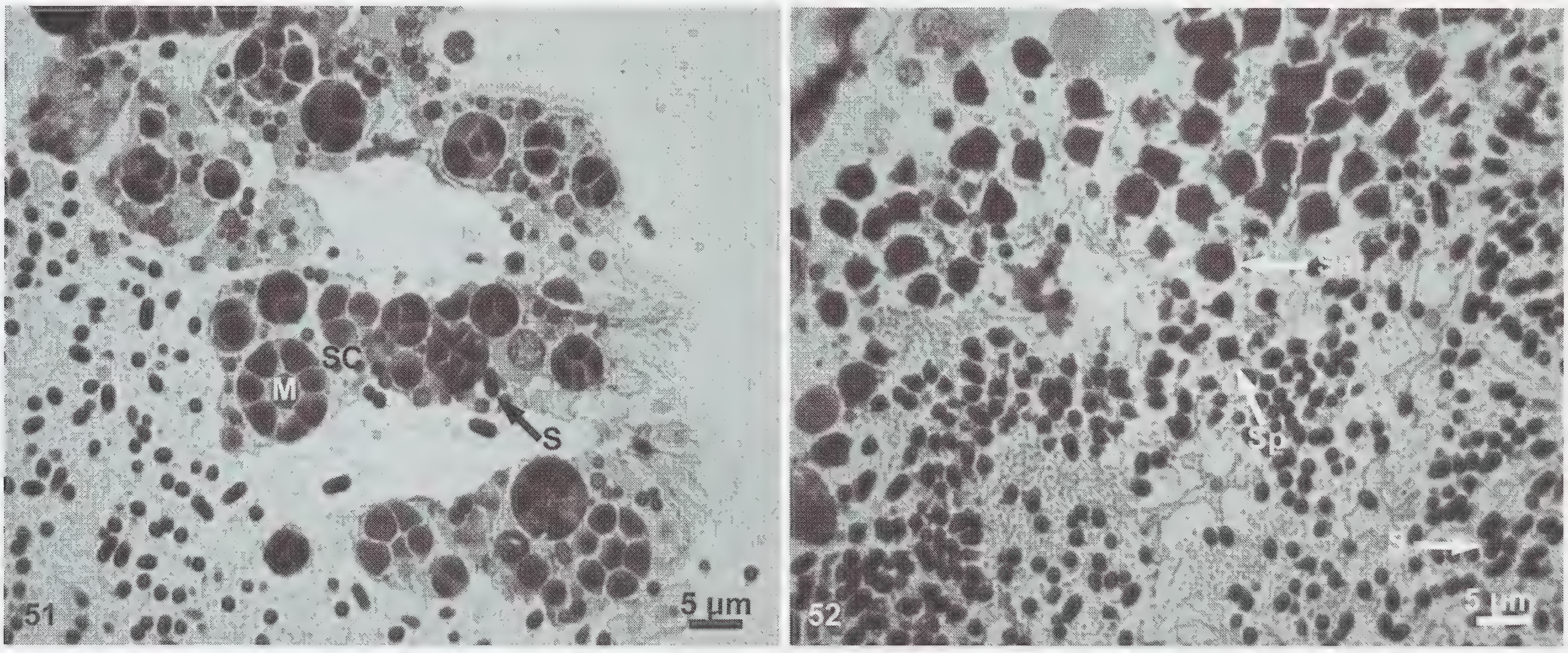
FIGS. 33–38. Atypical spermatogenic pathway cells and phagocytes common to both spermatogenic pathways. FIG. 33: Amorphous inclusion in Sertoli cell of atypical pathway; note the presence of nuclear-like material (arrows); FIG. 34: Apparent early breakdown stages of morulae into an amorphous inclusions; the amorphous inclusion on the right appears to be in an earlier stage of breakdown than the one on the left; note the presence of nuclear-like material (arrows); FIG. 35: Amorphous inclusions in close contact within Sertoli cell of atypical pathway; FIG. 36: Apparent transitional stage between morula and amorphous inclusion; FIG. 37: Phagocytes associated with an acinus wall; FIG. 38: Phagocytes associated with an acinus lumen.



FIGS. 39–44. Phagocytes and cells with calcium concretions common to both spermatogenic pathways. FIG. 39: Phagocyte engulfing a spermatozoon with its long, thin cytoplasmic extensions (arrows); note the nucleus with patches of condensed chromatin in its interior and lining its outer edge; FIG. 40: Phagocyte engulfing a lipid with its long, thin cytoplasmic extensions (arrows); FIG. 41: Lysosome engulfed by a phagocyte; the cytoplasmic extensions (arrows) encircle the lysosome; FIG. 42: Amorphous inclusion engulfed by a phagocyte; FIG. 43: Calcium concretions (arrows) engulfed by a phagocyte; vacuoles filled with electron dense material; FIG. 44: Calcium concretions (arrows) in cells nearby the male gonadal tissue.



FIGS. 45–50. Immunoelectron staining of July spermatozoa and concurrent presence of vitellogenic eggs and typical and atypical pathway cells in July. FIG. 45: Immunoelectron microscopy of July spermatozoon using the M*COX2e* (M mt genome encoded protein) antibody showing positive presence (gold balls, arrows) of the protein on the inner mitochondrial membrane; FIG. 46: Control spermatozoon for Fig. 45; FIG. 47: Immunoelectron microscopy of July spermatozoon using the F*ORF* (F mt genome encoded protein) antibody showing positive presence (gold balls, arrows) of the protein on the inner mitochondrial membrane; FIG. 48: Control spermatozoon for Fig. 47; FIG. 49: Vitellogenic egg, recognizable by its attached stock; FIG. 50: Presence of both typical and atypical pathway cells together in acinus in July.



FIGS. 51, 52. Atypical and typical spermatogenic pathway cells. FIG. 51: Atypical pathway Sertoli cell in acinus with spermatozoa in July; FIG. 52: Typical pathway cells with spermatozoa in acinus in July.

tebrates (but distinct from Sertoli cells), were noted in the male gonadal tissue from both the typical and atypical pathways. They were found associated both with the acinar walls (Fig. 37) and lumina (Fig. 38). These phagocytic cells were roughly rectangular in general outline and each contained a large nucleus with patches of condensed chromatin in its interior and lining the nuclear periphery (Figs. 38–43). Long, thin cytoplasmic extensions were observed enclosing a spermatozoon (Fig. 39), lipid droplet (Fig. 40), lysosome (Fig. 41), an AI (Fig. 42) and calcium concretions (Fig. 43). The calcium concretions were the only phagocytized material not observed in cells from the typical or atypical pathway. Concretions were seen in large quantities in cells nearby the male gonadal tissue (Fig. 44).

Immunoelectron Microscopy of Spermatozoa

Using single antibody experiments, some spermatozoa mitochondria were shown to possess the M*COX2* protein, encoded by the M mt genome, in their inner mitochondrial membranes (Figs. 45, 46), whereas the mitochondria from some other spermatozoa were shown to possess the F*ORF* protein, encoded by the F mt genome, in their inner mitochondrial membranes (Figs. 47, 48).

Overlapping Presence of Vitellogenic Eggs, Typical and Atypical Spermatozoa

Vitellogenic eggs, recognizable by their attached stalks (Fig. 49), were observed from May through October. Both typical and atypical spermatogenesis occurred simultaneously during this same time period (Figs. 50–52). Figures 50, 51, and 52 are from samples taken in July when fertilization takes place in this particular population of *V. ellipsiformis* (Chakrabarti et al., 2007, 2009).

DISCUSSION

In *Venustaconcha ellipsiformis*, spermatogenesis occurs along two distinct processes called the typical and atypical pathways. Spermatozoa produced by the two pathways were morphologically identical with a single acrosomal vesicle. While spermatozoa ultrastructure has been used to estimate phylogenetic relationships in bivalves, it has been suggested that acrosome structure should also be evaluated in relation to the amount of egg jelly necessary to dissolve prior to spermatozoon penetration. For example, brooding bivalves tend to exhibit less egg jelly presumably because the eggs are protected from environmental stresses and the univesicular acrosome likely provides adequate enzymes to dissolve the relatively small amount of jelly (Popham, 1979). *Venustaconcha ellipsiformis* is a long term brooding species (bradytictic) (van der Schalie & van der Schalie, 1963) and, as Popham (1979) noted, the structure of the acrosome in a species such as this could be more of a response to the functional aspects of the fertilization events noted above and thus have limited phylogenetic significance.

The developmental origin of morulae has long been an enigma as has that of atypical spermatozoa. No previous study in which morulae were reported (Heard, 1975; Kotrla, 1988, 1989; Haggerty et al., 1995; Byrne, 1998; Matos et al., 1998; Garner et al., 1999; Shiver, 2002; Cek & Sereflisan, 2006; Chatchavalvanich et al., 2006) has documented a source for the morulae. As Dohman (1983) pointed out, some studies of gastropod mollusks, such as *Epitonium tinctum*, *Bithynia tentaculata* and *Crepidula plana*, have indicated that spermatogonial cells were considered the source of atypical spermatozoa, but these atypical spermatozoa did not involve morulae and the resulting spermatozoa were not morphologically indistinguishable from typical spermatozoa as is the case in *V. ellipsiformis*. As stated previously, we propose that the origin of the morulae, and thus of the atypical pathway, is due to spermatogonial cells located within Sertoli cells. Thus, there appears to be a developmental connection between the typical and atypical pathways via the spermatogonial cells. The atypical pathway spermatogonial cells (2N) were indeed seen dividing into groups of spermatogonial cells that were enclosed within the Sertoli cells and the 1-celled morulae would be the analog of the primary spermatocytes (2N) of the typical pathway. As the spermatogonial cells continue to divide, 2, 4, 8, 16 and 32-celled morulae are produced. There are no cells that are morphologically similar to the secondary spermatocytes of the typical pathway in this scheme, but that does not preclude the possibility that some of the cells in morulae are analogues of secondary spermatocytes but with different morphologies. One possibility is that the cells in the morulae would initially undergo mitotic and subsequently meiotic divisions ultimately producing 32-celled morulae wherein each cell would mature into a haploid atypical spermatozoa. Alternatively, a hypothesis that the atypical pathway represents ameiotic spermatogenesis (Kotrla, 1989), producing diploid spermatozoa, cannot be ruled out at present. The future use of densitometric and/or fluorometric methods (e.g., Hardie et al., 2002) to estimate spermatozoon ploidy level would facilitate the evaluation of these two hypotheses. Nevertheless, in *V. ellipsiformis*, we interpret the morulae as containing 1, 2, 4, and the presumed 8, 16, or 32 cells as providing a strong inference that the cells in the morulae were dividing. A careful examination of Figure 1A in Matos et al. (1998) reveals that a similar interpretation is also justified for that study. Apparent stages of division were noted in *V. ellipsiformis* as was decondensed nuclear material typical of dividing cells. It appeared that cell division in morulae was not necessarily synchronous among the cells, as some cells were observed dividing when others were not, and mature spermatozoa were noted among cells within the same morula. Aggregates of atypical spermatozoa in membrane bound sacs (i.e., the membrane that previously surrounded a single morula) resulted within the Sertoli cells, and these spermatozoa eventually were released into the acinar lumen.

The observational evidence and logical reasoning for this hypothesis regarding the origin of the atypical spermatozoa is compelling. First, the spermatozoa must be generated from germ cells and the spermatogonial cells are the only known cells of this type available. Further, no observations were made of phagocytic activity by Sertoli cells, which could have led to the uptake of spermatids from outside the Sertoli cells. The possibility that spermatids from the typical pathway remained in acini after October and continued to form morulae until the following May also seems remote. Therefore, there is no evidence that the origin of morulae cells was from the typical pathway spermatids. There also is no evidence that the nuclei of the Sertoli cells were involved in the origin of the morulae cells. The Sertoli cell nuclei were never observed dividing in a manner similar to that observed in typical pathway spermatogonial cells. It is also notable that Sertoli cells apparently transitioned from containing many to few or no spermatogonial cells (Figs. 18, 19), which supports the contention that spermatogonial cells did continue to divide to create the morulae cells. Further, spermatozoa from the atypical pathway were initially observed inside membrane bound sacs in clusters which would be a logical expectation if they are developing from morulae cells also found inside a membrane. If further studies confirm the hypothesized transition of morulae cells from diploid to haploid, the morulae cells that are all currently referred to as spermatids may need to be divided into two groups with names referring to the diploid and haploid states such as “D morulae cells” and “H morulae cells”. The last set of haploid morulae cells prior to their maturation into atypical spermatozoa would be the true spermatids.

Chatchavalvanich et al. (2006), in a light microscopy study, also observed morulae in the unionoid bivalve *Hyriopsis bialatus* that were essentially identical to those found in *V. ellipsiformis.* Chatchavalvanich et al. (2006) proposed a similar atypical pathway of spermatozoa production via morulae for *H. bialatus* as we do for *V. ellipsiformis.* However, they called the original one-celled morula a spermatogonial cell and concluded that the cell types in the developing morulae included primary and secondary spermatocytes along with spermatids and spermatozoa. The morulae cells in the Chatchavalvanich et al. light micrographs appear remarkably similar to those observed at the light microscopy level in the present study. With the added benefits of electron microscopy in our study, however, it is clear that the cells within the morulae do not share characteristics with spermatogonial cells or primary and secondary spermatocytes but rather are morphologically very similar to spermatids. We suggest that the same is true of the morulae cells in Chatchavalvanich et al. (2006). With this difference in interpretation noted, however, we are in agreement with Chatchavalvanich et al. (2006) that spermatogonial cells are the likely progenitors of atypical spermatozoa and that atypical spermatozoa develop from spermatid cells in morulae. The presence of spermatozoa within the morula in Figure 23 provides substantial evidence that morulae cells do undergo development into spermatozoa, which is in agreement with conclusions and inferences from other studies (Heard, 1975; Kotrla, 1988; Haggerty et al., 1995; Matos et al., 1998; Garner et al., 1999; Shiver, 2002; Cek & Sereflisan, 2006; Chatchavalvanich et al., 2006).

Morulae consisting initially of spermatid-like cells were found in high numbers in Sertoli cells of the atypical spermatogenic pathway in *V. ellipsiformis.* Similar structures have been reported in other unionoid bivalve species (see Introduction). Shiver (2002) noted that morulae cells of *Lampsilis rafinesqueana* were precursors of spermatids and spermatozoa, while Jones et al. (1986) and Bloomer (1946) inferred the same for *Cucumerunio novaehollandiae* and *Anodonta cygnea*, respectively. Those depicted in *Prisodon alatus* (Matos et al., 1998) closely resemble the morulae observed in *V. ellipsiformis.* Matos et al. (1998) suggested that the Sertoli cells were somehow absorbing spermatids produced by a “natural spermatogenesis” and grouping them together inside a membrane. Further, Matos et al. (1998) hypothesized that microtubules in pseudopodia moved the morulae within the Sertoli cell and the spermatids then matured into spermatozoa. Matos et al. (1998) do not speculate on the specific origin of the spermatids or the function of the resulting spermatozoa and it is not clear what is meant by “natural spermatogenesis”. However, no pseudopodia were seen on the Sertoli cells of *V. ellipsiformis* and no engulfment of any type of material by Sertoli cells was observed. It is possible that the collections of spermatozoa in *V. ellipsiformis* represented phagocytized spermatozoa as was seen in the study of sea urchins (Reunov et al., 2004). However, as with the morulae, no engulfment or digestion of spermatozoa by Sertoli cells of *V. ellipsiformis* was observed similar to the phagocytic activity of the NP cells in the Reunov et al. (2004) report. On the contrary, in *V. ellipsiformis*, normal appearing spermatozoa were released into the acinar lumen during atypical spermatogenesis. Also, it seems unlikely that flagella would be carefully wound around the nucleus and mid-piece of spermatozoa during a phagocytic or engulfment event. Based upon the above evidence, it is concluded that Sertoli cells in *V. ellipsiformis* are not involved in phagocytosis of spermatids that form the morulae or spermatozoa in Sertoli cells.

Sertoli cells have been observed involved with phagocytic activity during spermatogenesis in many other molluscs (Syasina, 1992), but they were not found so associated in *V. ellipsiformis*. Because *V. ellipsiformis* male acini were cleared of debris, a search was made to locate alternative cells that could be performing this important process instead of Sertoli cells. There are other cells in the male acini, distinct from the Sertoli cells, which we interpret as phagocytes. Their general appearance was quite different from the Sertoli cells (Figs. 15, 19 vs. Figs. 37, 38). These presumed phagocytic cells also demonstrated the classic stages of phagocytosis, for example, endocytosis of cellular debris using long cytoplasmic extensions and apparently containing digested cellular materials and organelles. No cells resembling hemocytes (Dundee, 1953; Higashi, 1980; Wen et al., 1994; Burkhard et al., 2009), which also can function as phagocytes, were seen in the acini of *V. ellipsiformis*.

While there are atypical spermatogenic pathways in a variety of organisms (Cook & Wedell, 1999; Ferraguti et al., 2002; Till-Bottraud et al., 2005; Paccagnini et al., 2006; Immler, 2008), including molluscs (Buckland-Nicks & Chia, 1986; Jespersen et al., 2001; Buckland-Nicks & Thompkins, 2005), a pathway including AI in Sertoli cells has not been reported. The role of Sertoli cells in destruction of materials by lysosomal activity is well documented in molluscs (Buckland-Nicks & Chia, 1986; Pipe, 1987; Dorange & LePennec, 1989; Sousa et al., 1989;

Rocha & Alzevedo, 1990; Syasina, 1992; Eckelbarger & Davis, 1996; Kiyomoto et al., 1996; Erkan & Sousa, 2002). It is possible that, in *V. ellipsiformis*, the AIs are organelles resulting from lysosomal activity, and the observed transition of the appearance of the material inside them occurs as sperm morulae break down into or are digested by the AIs. Under this scenario, the peculiar yellowish brown color (using the Feulgen stain) of the AIs could be indicative of digesting DNA from the morulae cells. Apparent steps of morulae undergoing a breakdown process were observed many times. Figures 32–36 are particularly suggestive of various phases of morulae digestion, especially when comparing Figure 21, an intact morula, to Figures 33 and 34, where morulae appear to be undergoing the digestion process. The close proximity of sperm morulae and AIs with lipids could indicate that the lipids provide energy for this process, while the nearby lysosomes could contribute enzymes for breaking down the morulae. Under this interpretation, AIs could be considered a type of secondary lysosome. It is also possible that, as the morulae degenerate into AIs, the individual AIs combine together. This would mean that the AIs were undergoing a fusion event as is typical of lysosomes (Karp, 1999) and is supported by the observed close special association between AIs.

Phagocytic and lysosomal activities have been reported as important in clearing out the acini of unused spermatozoa in preparation for upcoming spermatogenesis during the yearly reproduction cycles in sea urchins (which are deuterostomes and thus distantly related to bivalves) (Reunov et al., 2004). Reunov et al. (2004) observed accessory cells called nutritive phagocytes (NPs) that engulfed spermatozoa. The individually phagocytized spermatozoa then aggregated into larger heterophagosomes, which were digested into bodies referred to as remnant bodies. Remnant bodies were eventually destroyed, leaving a vacuolar space that disappeared through cytoplasmic compression. The remnant bodies are morphologically very similar to the AIs of the present study and appear to be formed by a similar process involving lysosomal activity. However, there are some important differences between the formation of the remnant bodies in sea urchins and the AIs in *V. ellipsiformis*. First, the AIs could be a product of morulae digestion whereas the remnant bodies were involved in spermatozoa digestion. Second, the spermatozoa were phagocytized while there was no evidence of a similar process with the morulae, that is, no long, thin extensions commonly associated with phagocytes were observed on Sertoli cells. Finally, there was no evidence of AI destruction and reabsorption within the cells. Rather, the AIs were released intact into the acinar lumen as the Sertoli cells broke down and some AIs were seen being absorbed by phagocytes that were structurally distinct from the Sertoli cells. If the AIs in *V. ellipsiformis* are digested morulae, then a substantial percentage of sperm morulae created in the atypical pathway are broken down. Perhaps the contents of some of the morulae cells are recycled as nutrients within the Sertoli cells. A similar role of providing nutrients to developing spermatozoa has been reported for Sertoli cells in other molluscs (Pipe, 1987; Dorange & LePennec, 1989; Syasina, 1992). Alternatively, some morulae could be defective and thus could be selected for destruction as occurs in triploid mussel spermatocytes that do not mature (Kiyomoto et al., 1996).

The typical and atypical spermatogenic pathways overlapped in *V. ellipsiformis* from May to October. Because the spermatozoa produced by the two distinct pathways were morphologically indistinguishable, it was impossible to tell which pathway produced them unless the spermatozoa were located inside the clusters of the atypical pathway. It is important to note that vitellogenic eggs were also present in this same May to October time frame. At present, the only documented fertilization event for this species in Michigan occurs in late July. It is possible that additional fertilization events occur in October, for example, in more southern *V. ellipsiformis* populations, but not in Michigan due to the colder fall temperatures. Nevertheless, the atypical pathway might be active year round because it requires less energy and can withstand the colder temperatures of fall and winter or because it has its more of its own energy source in the recycling of nutrients via the breakdown of the morulae into AI which then act as an energy reservoir. This hypothesis requires that atypical spermatozoa have a relatively long functional longevity within testes.

There are several hypotheses that could account for the two spermatogenic pathways in unionoid bivalves. One is that only spermatozoa from the typical pathway are responsible for fertilization and the atypical spermatozoa have some other role. The positive stain for DNA in atypical spermatozoa rejects the hypothesis that they are apyrene, that is, devoid of DNA, as is the case with the atypical spermatozoa of many organisms. This further supports the hypothesis that atypical spermatozoa can fertilize eggs. The morphological similarity between the typical and atypical spermatozoa also lends credence to this hypothesis, as does the presence of the dual spermatogenic pathways in all unionoid families (Heard, 1975, unpublished; Kotrla, 1989). The latter point suggests that these pathways were both operative in the unionoid common ancestor (> 200 million years ago) and that they both persisted to the present in all familial lineages. Overall, the above evidence argues strongly for the fertilization functionality of atypical spermatozoa in unionoids while rejecting a nonfunctional hypothesis.

Given the above evidence supporting the fertilization functionality of atypical spermatozoa, the pivotal question remains as to why there are two spermatogenic pathways in *V. ellipsiformis* and in unionoids generally. One hypothesis is that production of atypical spermatozoa during times of the year when there is no fertilization (November to early May) is a means to keep the germ tissues active at a low level so that they can be readily reactivated at a high rate when conditions are appropriate for fertilization. This hypothesis implies that atypical spermatozoa are not used for fertilization. However, this hypothesis can be rejected by the observations that atypical and typical spermatogenesis occur simultaneously in *V. ellipsiformis*. Furthermore, the long term maintenance of ultrastructural identity between these two pathways' end products would not be expected due to relaxed selection on the atypical pathway (see below).

A second hypothesis is that the spermatozoa from one of the two pathways play only a structural role in the formation of the spermatozeugmata (the spherical aggregates of spermatozoa released by unionoid males; Lynn 1994) while the other pathway's spermatozoa are responsible for actually fertilizing the eggs. However, if the spermatozoa from one pathway are only structural, why would natural selection maintain the presence of DNA, acrosomes and identical ultrastructure in the "structural" spermatozoa for > 200 million years? Rather, under the structural hypothesis, it would be expected that unionoid spermatozoa produced by the "structural" pathway would have attained the apyrene condition after > 200 million years of phylogenesis.

A third hypothesis for the long-term maintenance of two spermatogenic pathways is that atypical spermatozoa are produced and released prior to the production and release of typical spermatozoa in order to communicate with females to facilitate synchronized gamete maturation, and that only typical spermatozoa participate in fertilization. Under this hypothesis, one would predict that (1) atypical spermatozoa would always be produced/released before the typical spermatozoa and (2) natural selection would eventually reduce the energetic expenditure on atypical (i.e., non-fertilizing) spermatozoa by eliminating the acrosome, DNA, etc. However, given the synchronized occurrence of typical and atypical spermatogenesis in *V. ellipsiformis* and the long term ultrastructural identity of these two pathways' end products, the "communication" hypothesis can be rejected.

A fourth hypothesis is that the two distinct pathways' spermatozoa are involved in the maintenance of dioecy (i.e., separate sexes) and 50 : 50 sex ratios in populations of unionoid bivalves. Heard (1975) suggested that the atypical pathway in *Utterbackia imbecillis* (always hermaphroditic; W. R. H., personal observation [but see Heard, 1975]) is greatly reduced relative to the other anodontine species (all dioecious) he examined. This observation is consistent with the hypothesis that both spermatogenic pathways are required for the maintenance of dioecy and 50 : 50 sex ratios in unionoids. The IEM-based inference reported herein, that a spermatozoon contains either F- or M-genome-bearing mitochondria, also corroborates this hypothesis due to the recent discovery that the genetic transmission of both F and M mt genomes is required for the maintenance of dioecy in unionoids, i.e., with M mt genomes responsible for maleness (Breton et al., 2011). Thus, the above observations (1) reject the hypothesis that only M mt genome-bearing spermatozoa effect fertilization in species with DUI (Venetis et al., 2006) and (2) are consistent with the hypothesis that the dual spermatogenic pathways in unionoids are a specialization to produce two types of spermatozoa, that is, those with M genome-containing mitochondria and those with F genome-containing mitochondria. If, as proposed earlier (Breton et al., 2011), the F and M mt genomes directly participate, through interactions with the nuclear genome, in the genetic regulatory network specifying dioecy in unionoid bivalves, the inference can be made that spermatozoa with F genome-containing mitochondria are less likely to facilitate maleness and could thus participate in the maintenance of 50 : 50 sex ratios in bivalve populations. In other words, it is hypothesized that males in male-biased populations produce a larger proportion of spermatozoa with F genome-containing mitochondria than males in female-biased populations. Because the presence of both M mt genomes and the atypical spermatogenic pathway are derived character states, it is further hypothesized that the atypical pathway produces spermatozoa with M genomes while the typical pathway produces spermatozoa with F genomes. Nevertheless, the long-term co-occurrence of two highly unusual phenomena in unionoid bivalves, that is, (1) dual spermatogenic pathways yielding morphologically identical spermatozoa and (2) high fidelity DUI (Hoeh et al., 2002), strongly suggests that the two phenomena are causally linked (Zouros, 2000). Further research is required to test the "maintenance of dioecy and 50 : 50 sex ratios" hypothesis and, if it is confirmed, determine the mechanisms underlying these two unusual phenomena.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Kathleen Pelkki for the line drawings and Robert Huber, Geron Johnson, Kim Anklam, Ashley Verner, Kelly Buxman, Erin (Griffore) Arnst and Jennifer Watson for assistance in specimen preparation. We thank Mark Gordon for comments on previous versions of the manuscript. Funding for this study was supplied by grants to S. Shepardson from the Saginaw Valley Foundation and the Saginaw Valley State University Professional Growth Fund and NSF Grant DMI-0619270.

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Revised ms. accepted 28 August 2012